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Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants

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Summary

Different transgenic tobacco lines down-regulated for either one or two enzymes of the monolignol pathway were compared for their lignin content and composition, and developmental patterns. The comparison concerned CCR and CAD down-regulated lines (homozygous or heterozygous for the transgene) and the hybrids resulting from the crossing of transgenic lines individually altered for CCR or CAD activities. Surprisingly, the crosses containing only one allele of each antisense transgene, exhibit a dramatic reduction of lignin content similar to the CCR down-regulated parent but, in contrast to this transgenic line, display a normal phenotype and only slight alterations of the shape of the vessels. Qualitatively the lignin of the double transformant displays characteristics more like the wild type control than either of the other transgenics. In the transgenics with a low lignin content, the transformations induced other biochemical changes involving polysaccharides, phenolic components of the cell wall and also soluble phenolics. These results show that the ectopic expression of a specific transgene may have a different impact depending on the genetic background and suggest that the two transgenes present in the crosses may operate synergistically to reduce the lignin content. In addition, these data confirm that plants with a severe reduction in lignin content may undergo normal development at least in controlled conditions.

Keywords: lignin, lignification, transgenic tobacco, plant development, cinnamoyl CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD).

Introduction

Lignin biosynthesis has been successfully manipulated using various target genes (Baucher *et al.*, 1998; Grima-Pettenati and Goffner, 1999). Research programmes, motivated by applied objectives, aim to modify lignocellulosic materials currently used in the pulp and paper industry in order to improve pulp yields and decrease energy consumption and the environmental pollution associated with biomass processing. As woody species generally take longer to transform genetically than herbaceous plants, exploratory experiments have been frequently performed on model systems, such as tobacco, due to their simplicity of genetic manipulation and short regeneration cycles.

At the present time, several genes involved in the upstream steps (*4 cl*) or downstream steps (*ccr* and *cad*)

of lignin monomers biosynthesis and in the methylation of these monomers (*comt, ccoaomt and f5h*) have been shown to be interesting targets for lignin modification. The resulting data have demonstrated the high metabolic plasticity of the lignification process (Boudet, 1998; Ralph *et al.*, 1998; Sederoff *et al.*, 1999), the potential application in the pulp industry for some of the engineered plants (Baucher *et al.*, 1996; Lapierre *et al.*, 1999) and the complexity of the lignin biosynthetic pathway, which appears to function as a grid with enzyme isoforms, alternative pathways and tight regulatory interactions between specific branches (Li *et al.*, 2000).

In the context of lignin genetic engineering and for practical applications, plants with an induced decrease in lignin content should display a normal development. Unexpectedly, recent results have shown, both in tobacco (Zhong *et al.*, 1998) and in *populus* (Hu *et al.*, 1999; Zhong *et al.*, 2000), that dramatic decreases in lignin content do not necessarily adversely affect the growth of the plants. In contrast, our results on CCR down-regulated tobacco plants (Piquemal *et al.*, 1998) have shown that a strong reduction in lignin content may be associated with a profound alteration of development.

In order to understand these apparently contradictory effects and to more finely tune the lignin profiles, additional studies are necessary. It is indeed important to know if the global reduction of lignin *per se* or other secondary effects resulting from lignification genes down-regulation have negative developmental impacts. One strategy to obtain new lignin patterns in plants is to combine the effects of more than one transgene in a given individual transformant. The integration of multiple transgenes is indeed a prerequisite for manipulating complex biosynthetic pathways and agronomic characteristics in plants. Several experimental approaches are available for this purpose including repetitive insertions, transfer of multiple genes within one and the same construct, and multiple gene co-integration (Chen *et al.*, 1998). Various examples of successful multiple transgene integration and expression have already been reported (Ma *et al.*, 1995; Nawrath *et al.*, 1994; Ye *et al.*, 2000). Using repetitive insertions, Zhong *et al.* (1998) recently obtained transgenic tobacco plants with a reduction in both CCoAOMT and COMT, two methylation enzymes in the lignin pathway.

In previous studies we have obtained tobacco plants independently down-regulated in CAD and CCR (Halpin *et al.*, 1994 and Piquemal *et al.*, 1998). The CCR down-regulated plants had lower lignin levels than controls. The extractability of the lignin polymer was improved for CAD down-regulated plants without changes in the actual lignin content. Lignin structural differences were elucidated by NMR (Ralph *et al.*, 1998). CAD-deficiency resulted in the incorporation of significant levels of both coniferyl and sinapyl aldehydes into the polymers, resulting in new 8-O-4-cross-coupled structures (Kim *et al.*, 2000). CCR-deficient plants had lignins more reminiscent of wound-response products (Ralph *et al.*, 1998); tyramine hydroxycinnamates were major structures integrated into the polymer. In order to explore the potential for combining the advantages of lower lignin contents (provided by CCR down-regulation) and improved lignin extractability via structural changes (effected by CAD down-regulation), we envisaged obtaining double transformants by crossing individual homozygous CCR (CCR.H) and CAD (CAD.H) down-regulated lines. The resulting hybrids, bearing the two antisense transgenes, exhibit interesting characteristics: in spite of having about 50% of the lignin content of the wild type they show normal development and have a good potential for pulping applications.

These data highlight the specific phenotype resulting from the down-regulation of multiple genes of the monolignol biosynthetic pathway.

Results

Enzyme activities and lignin content of single and double transformants

Double transformants (Dt) resulted from the crossing in both directions of homozygous lines for the *ccr* or the *cad* antisense transgenes. *ccr* tobacco cDNA was initially characterized by Piquemal *et al.* (1998) and *cad* by Knight *et al.* (1992). The two homozygous lines initially called B3.1 (Piquemal *et al.*, 1998) and J48.7 (Halpin *et al.*, 1994) were renamed in this paper for the sake of clarity CCR.H and CAD.H and the two crosses were as follows: (Dt 1a: CAD.H♀x CCR.H♂ and Dt 1b: CCR.H♀x CAD.H♂). In all cases, the occurrence of both transgenes in the hybrids was confirmed by PCR (data not shown).

In one of the experiments corresponding to the crossing of homozygous lines (Dt 1a), we measured the amount of transcripts, the enzyme activities, and analysed quantitatively and qualitatively the lignin of the hybrid and of the homozygous parent lines. In parallel, the wild type and the hemizygous lines CCR.h and CAD.h resulting, respectively, from the backcross of the CCR.H and CAD.H lines with the wild type were also analysed.

Northern Blot experiments confirm that the introduction of the antisense *cad* cDNA reduces the expression of the corresponding resident *cad* gene in CAD.H, CAD.h and double transformant (Dt 1a). There is also a pronounced decrease of *ccr* transcripts in the transgenic lines containing the antisense *ccr* cDNA: CCR.H, CCR.h and double transformant (Dt 1a) (data not shown).

Table 1 reports the enzyme activities and the lignin content and composition of the different tobacco genotypes. The CCR and CAD activities strongly reduced in the CCR.H and the CAD.H lines, respectively, are also reduced

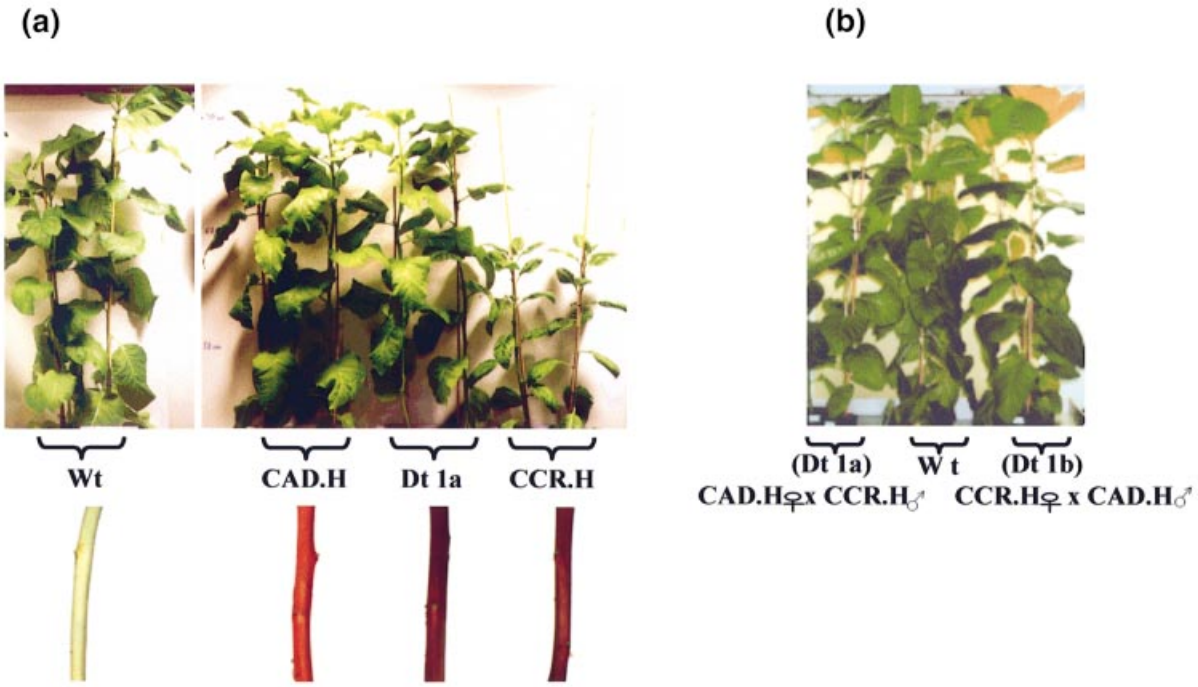


Figure 1. Phenotypes of 2.5-month-old-tobacco plants corresponding to wild type, double transformant CAD/CCR (Dt 1a and 1b) and both parents (CCR.H plant) and (CAD.H plant) with colour of the xylem after removing epidermis and cortex. (a) and (b) correspond to two different experiments.

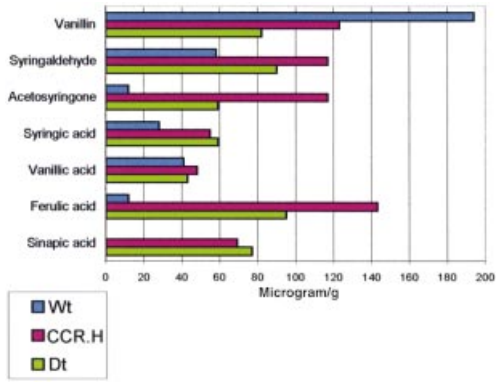


Figure 4. Individual amounts of cell wall alkali-released phenolics from different tobacco lines.
GC–MS analyses of the alkali-released phenolics were performed on CWR from control plant, CCR antisense parent line (CCR.H) and Dt 1a: double transformant CAD/CCR. Quantitative determinations were deduced from calibration with correspondence compounds and with reference to an internal standard (C22).

but to a lower extent in the hybrid genotype. The typical lignin profiles of CCR and CAD down-regulated plants were found for the two parents. CCR down-regulation reduced the lignin content which was not observed for the CAD down-regulated plants. The CCR.h line exhibited a limited reduction in lignin content when compared with the CCR.H line suggesting a gene dosage effect. Surprisingly, this potential correlation between the number of alleles of the transgene and the extent of lignin reduction was not found for the hybrid plants (Dt 1a) for which the decrease in lignin is close to that observed for the CCR parent. Similar trends were observed for the opposite sense of crossing (Dt 1b) (data not shown). These results may indicate a synergistic effect between the two transgenes in controlling the carbon flux towards lignin synthesis.

Plant morphology

The morphology and size of the parent plants, the hybrids and the wild-type grown for 2.5 months in a culture room and observed before flowering are shown in Figure 1. In CCR.H down-regulated plants, we observed a strong reduction in plant size, a loss of apical dominance, an abnormal shape of the leaves with necrotic spots, and brown coloration of the xylem (Figure 1a). Normal plant size and morphology and a pink–red colouration of the xylem was noted for the CAD.H down-regulated plants (Figure 1a). The double transformants (Dt 1a and 1b) (Figure 1a and b) exhibited a size and morphology similar to the control with, however, a brownish coloration of the xylem resembling the CCR.H parents. The plant hemizygous for the *ccr* transgene (CCR.h) presents characteristics between those of the CCR.H line and the control (data not shown).

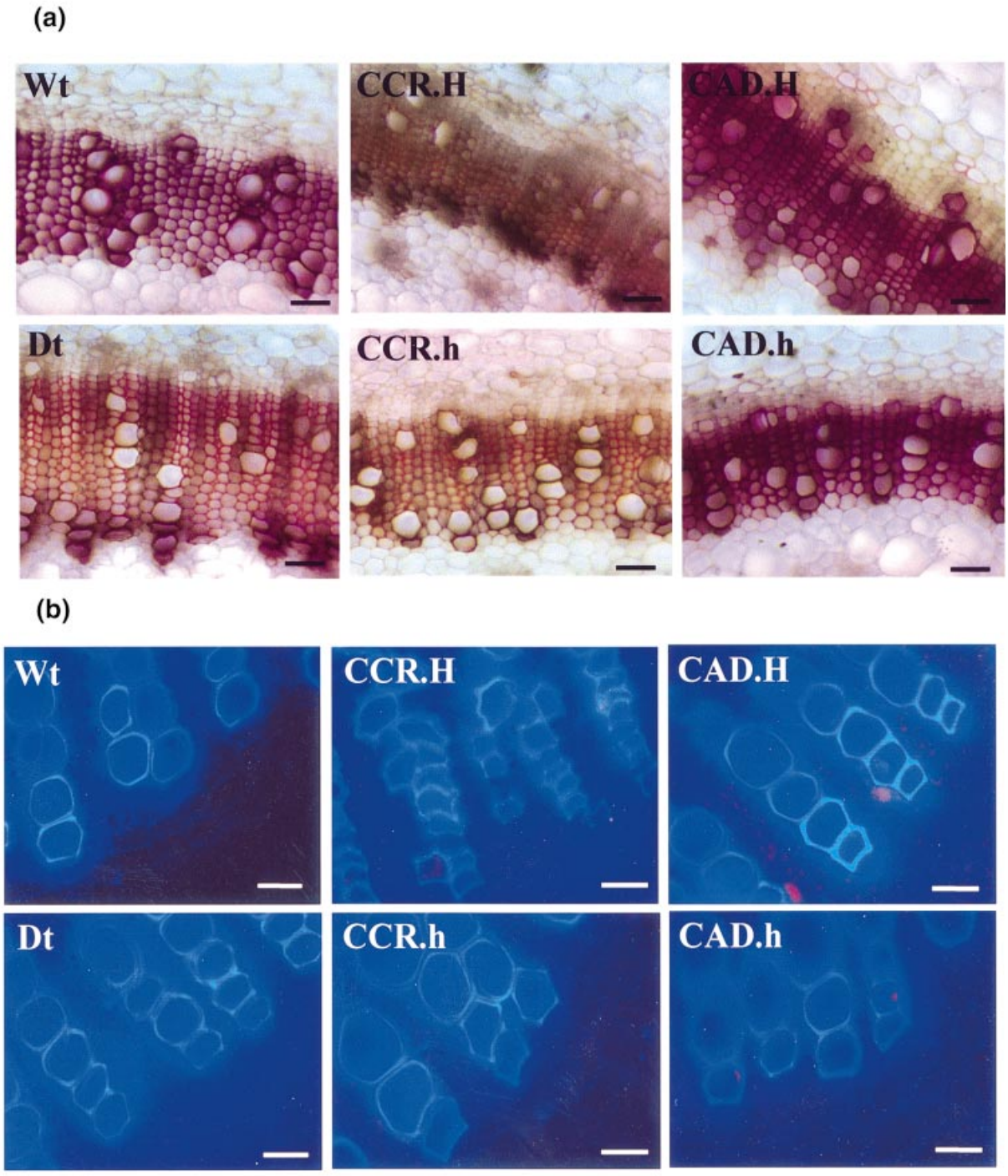


Figure 2. Microscopic inspection of liquified tissues in different tobacco lines.
(a) Transverse stem sections of the different tobacco lines after Phloroglucinol-HCl staining. Hand-made sections at the base of the stems are shown after phloroglucinol staining for the six following tobacco lines, Wt: control; CCR.H and CCR.h: CCR antisense lines, respectively, homozygous and hemizygous for the transgene; CAD.H and CAD.h: CAD antisense lines, respectively, homozygous and hemizygous for the *cad* transgene and Dt 1a: double transformant CAD/CCR. The black bar represents 50 μm.
(b) Natural autofluorescence of the lignified tissues seen on transverse petiole sections of different tobacco lines. Petiole sections for the same six tobacco lines are observed using epifluorescence microscopy. Vessels are seen under blue excitation (excitation filter BP 450–490 nm, suppression filter LP 520 nm) (Bar = 50 μm).

Table 1. Enzyme activities, lignin content and composition for several transgenic tobacco lines. CCR and CAD enzyme specific activities were measured on soluble protein extracts and the lignin content and composition were determined on CWR (Cell Wall Residue) by the Klason method and gas chromatography of lignin thioacidolysis products, respectively. Analyses were performed on the base of the stem for 2.5-month-old tobacco grown in culture-room conditions. For each line, 5 individual plants were pooled and the results represent the mean ± standard error of 4 independent extractions and corresponding enzyme activity measurements on the pooled material. In the same way, for Klason or GC thioacidolysis analyses the values represent the mean and standard error of four independent analyses on the pooled sample. 100% values for the control were, respectively, 68 pkat.mg^{-1} protein for CCR activity and 103 pkat.mg^{-1} protein for CAD.

	Relative enzyme activities		Lignin content (Klason)	G units	S units		S + G units	S + G units
	% CCR	% CAD		(μmoles. g^{-1} CWR)		S: G ratio	(μmoles. g^{-1} CWR)	(μmoles.g^{-1} Klason lignin)
Wild-type	100 ± 12.2	100 ± 5.8	22.2 ± 0.4	127.4 ± 3.8	116.1 ± 4.6	0.91 ± 0.01	243.5 ± 3.4	1096.8 ± 15.3
CCR.H (homozygous parent)	3 ± 1.6	115 ± 7.2	10.7 ± 0.5	18.9 ± 0.4	61.1 ± 2.3	3.22 ± 0.09	80.0 ± 2.6	747.6 ± 24.3
CCR.h (hemizygous plant)	11 ± 2.5	130 ± 8.2	15.1 ± 0.6	59.9 ± 0.5	84.1 ± 2.0	1.40 ± 0.03	144.0 ± 2.2	953.6 ± 14.5
CAD.H (homozygous parent)	100 ± 3.6	5.5 ± 1.2	21.1 ± 0.3	120.7 ± 4.5	49.6 ± 1.3	0.41 ± 0.01	170.4 ± 5.5	807.6 ± 26.1
CAD.h (hemizygous plant)	100 ± 1.8	64 ± 3.1	22.1 ± 0.7	130.5 ± 3.8	100.6 ± 5.8	0.77 ± 0.03	231.1 ± 9.2	1045.7 ± 41.6
CAD.H × CCR.H. (Dt 1a)	32 ± 2.6	12 ± 1.3	11.8 ± 0.4	38.0 ± 0.8	95.4 ± 1.2	2.51 ± 0.03	133.4 ± 2.0	1130.5 ± 16.9

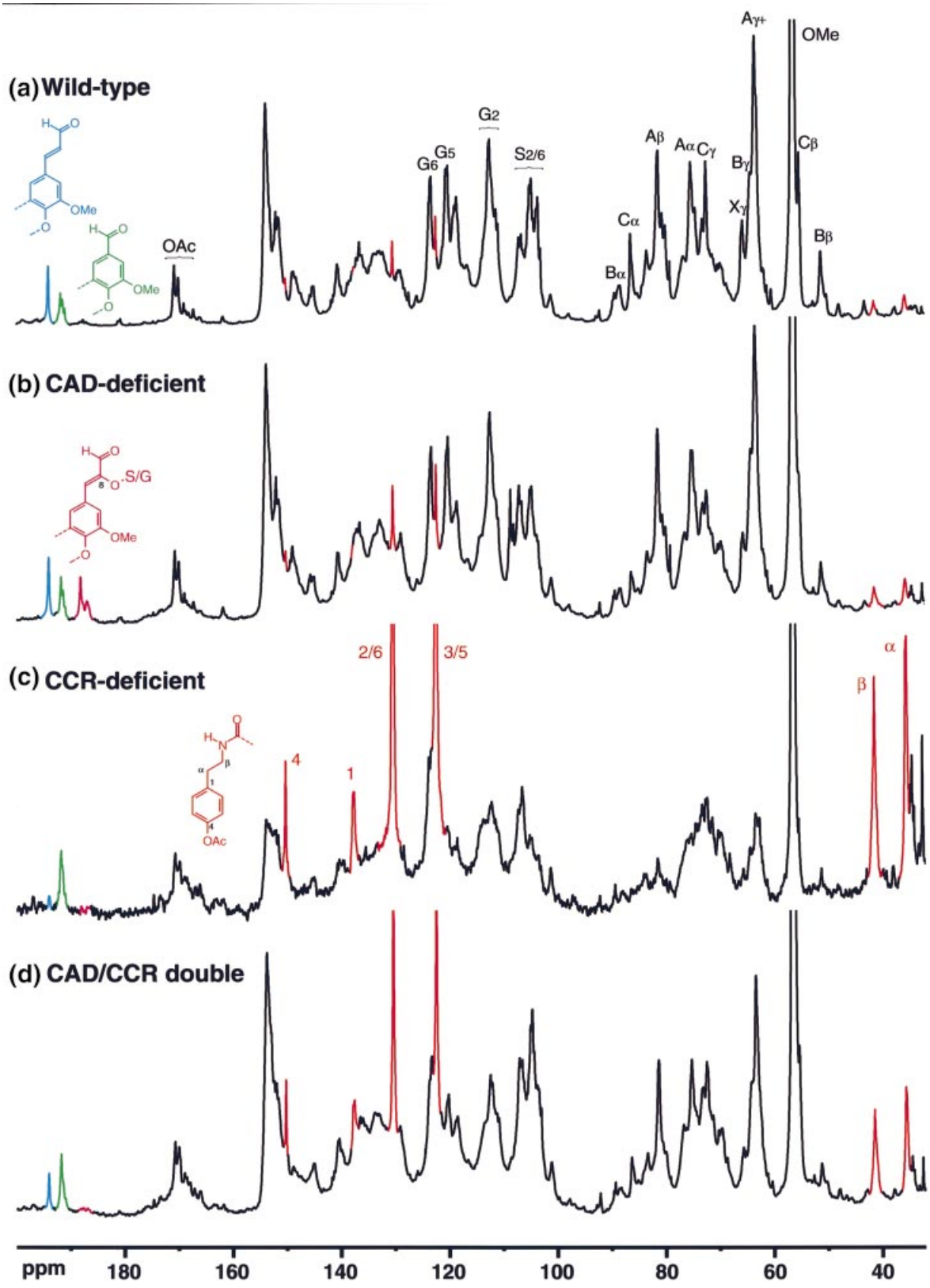
Histochemical inspection of the xylem cells in the different transgenic lines

Figure 2a reports a histochemical survey of the different transgenic lines using the Wiesner reagent (phloroglucinol–HCl) which specifically stains native lignins due to the formation of a purple chromophore between lignin hydroxycinnamaldehyde end-groups and phloroglucinol. As expected, relative to the control, both CCR depressed lines, either homozygous (CCR.H) or hemizygous (CCR.h) for the transgene, displayed reduced staining of the xylem area due to their lower lignin content. As noted from the NMR studies below, the lignins also contained fewer cinnamaldehydes relative to simple benzaldehydes. In contrast, CAD down-regulated plants CAD.H or CAD.h exhibited a slight increase in staining due to the increased proportion of cinnamaldehydes in their lignins (Yahiaoui *et al.*, 1998). The situation is more complex for the double transformant. It stained more intensely than CCR.H suggesting a higher content of cinnamaldehyde groups with its reduced lignin level. However, elevated aldehyde levels were not seen by NMR in the isolated lignins, although traces of the new 8-O-4 coupled structures (typical of CAD deficiency) were detectable (magenta peaks).

A more detailed analysis of vessel elements which exhibit a high spontaneous fluorescence under U.V. light due to their relative enrichment in G units (Musha and Goring, 1975) was performed on the petioles of the different transgenic lines (Figure 2b). As already shown for the stems of tobacco plants with a strong CCR down-regulation (Piquemal *et al.*, 1998) xylem vessels of the homozygous CCR line are collapsed and dramatically deformed (Figure 2b). In contrast, the vessels of the other transgenic lines are relatively similar to the wild type. Despite a dramatic decrease in the lignin content of the stem, the xylem vessels of the double transformant (Dt 1a) are not significantly altered.

Lignin composition

via analytical thioacidolysis. Some structural features of wild type and transgenic lignins are reported in Table 1, which summarizes the data obtained from the analysis of thioacidolysis lignin-derived monomers. In agreement with previous results (Halpin *et al.*, 1994; Piquemal *et al.*, 1998), the opposite effect of CCR versus CAD down-regulation on the thioacidolysis S: G ratio was observed. The thioacidolysis S and G monomers specifically originate from lignin units involved in labile ether bonds, that is from Ar-CHOH-CHOAr-CH_2OH units (with Ar = G or S ring). These arylglycerol-β-aryl ether structures will be referred to as 'uncondensed structures' for the sake of simplicity. The common effect of the two antisense transgenes when employed individually is the reduction of thioacidolysis


(a) Wild-type
OAc
G6
G5
G2
S2/6
Bα
Cα
Aβ
Aα
Cγ
Bγ
Xγ
Aγ+
OMe
Cβ
Bβ
(b) CAD-deficient
S/G
OMe
(c) CCR-deficient
4
1
2/6
3/5
β
α
OAc
(d) CAD/CCR double
ppm
180
160
140
120
100
80
60
40

yield, indicative of a higher content of resistant interunit bonds in the lignin. The S: G decrease induced by CAD deficiency reveals that uncondensed S structures are more affected than their G analogues. In contrast, the S: G increase observed in CCR.H and, to a lower extent, in CCR.h shows that CCR down-regulation more specifically reduces the lignin content in G uncondensed structures and likely induces the formation of additional carbon–carbon interunit bonds involving more specifically G lignin units. As discussed below, polymerization of significant levels of ferulates into these polymers also results in a lower release of traditional thioacidolysis monomers. In the double transformant and with regard to the S: G ratio, the impact of CCR silencing seems to predominate over that of CAD. In contrast the thioacidolysis yield seems to be restored to the control level. This could be largely due to the lower incorporation of ferulates into lignins and to the lower alteration of lignin structure (see following subsection ''via NMR'').

via NMR. NMR is particularly valuable for deducing more detailed structural aspects of soluble lignin fractions (Ralph *et al.*, 1999). Unfortunately in the case of tobacco, the yields of isolated soluble lignins are low (see Experimental procedures section). Nevertheless, if an extract from a transgenic line differs substantially from that of the control, it is logical that such structural differences will be present in the whole polymer at some level.

Figure 3 shows 1D ^{13}C NMR spectra of soluble lignins from the wild-type control, the CAD.H and CCR.H down-regulated transgenics, and the double transformant (Dt 1a). As has been noted previously (Ralph *et al.*, 1998), wild-type tobacco lignins are fairly typical angiosperm lignins composed of syringyl and guaiacyl units, Figure 3(a). The normal incorporated aldehydes, of both the hydroxycinnamaldehyde and the derived hydroxybenzaldehyde type (from 190 to 195 p.p.m.: blue and green peaks on the Figure 3) are at slightly higher levels than seen in most hardwoods or other dicots. Another feature, not strikingly evident in Figure 3(a) until compared with the CCR.H deficient transgenics, is the appearance of tyramine units. Such units are present as amides to hydroxycinnamates, particularly ferulate (Ralph *et al.*, 1998). Ferulates are intimately incorporated into the polymer by radical coupling reactions at their 4-O-, 5-, and 8-positions to form an array of complex structures (Ralph *et al.*, 1992) among which the ferulate moieties are difficult to identify. The tyramine units are terminal, remain free phenolic, and are relatively structurally invariant, so are seen as sharp peaks (Figure 3c).

The CAD.H down-regulated transgenic, Figure 3(b), shows the typical signature of CAD deficiency in angiosperms, albeit at lower levels than observed in a prior antisense-CAD transgenic (Ralph *et al.*, 1998). New aldehyde peaks corresponding to hydroxycinnamyl aldehydes 8-O-4-coupled to both guaiacyl and syringyl units, are prominent aldehyde peaks. The tyramine levels seem to be marginally elevated, and the relative guaiacyl content is a little higher, as noted from the thioacidolysis data.

Lignin from the CCR.H down-regulated transgenic could be extracted in only a very low yield. As in a previous study, it is characterized by incorporation of substantial amounts of tyramine units (Ralph *et al.*, 1998). In this case

Table 2. Weight percentage of the various neutral sugars recovered from acid hydrolysis of cell wall residue. Sulphuric acid hydrolysis followed by GC analysis of the released sugars (analysed as their alditol acetates) was performed on three different tobacco lines (Wild type, CCR.H depressed line and Dt 1a: CAD/CCR double transformed line). Three repetitions were performed for each line and the average value ± standard error are given in this table.

	% neutral sugar				
Tobacco lines	Arabinose	Xylose	Mannose	Galactose	Glucose
Wt	0.64±0.03	12.12±1.10	1.60±0.09	0.65±0.04	23.70±1.13
CCR.H	0.58±0.08	16.02±0.29	1.47±0.04	0.68±0.02	38.26±2.05
Dt 1a	0.58±0.01	16.25±0.03	1.64±0.07	0.62±0.05	30.83±0.46

Figure 3. NMR spectra (1D ^{13}C) of lignins of different transgenic tobacco plants.
NMR spectra (1D ^{13}C, 90 MHz) of lignins isolated from (a) wild type (b) CAD.H down-regulated line (c) CCR.H down-regulated line and (d) CAD/CCR-doubly transformed tobacco (Dt 1a). Signature aldehydes (cyan for cinnamaldehydes, green for benzaldehydes and magenta for the 8-O-4-coupled cinnamaldehydes) and tyramine units (red) are coloured for ready identification, and some of the prominent peaks and peak regions are labelled in the control; G = general guaiacyl aromatic ring (positions 1–6 are conventionally labelled with the sidechain attachment position as 1; sidechain α, β, and γ with the aromatic ring attached to C-α), S = general syringyl unit (labelled analogously), A = β-aryl ether (β-O-4), B = phenylcoumaran (β-5), C = resinol (β-β), X = cinnamyl alcohol endgroup; α,β,γ refer to the sidechain positions of units A-C, X (α is the sidechain position attached to the aromatic ring); OMe = methoxyl carbons; OAc = acetate carbonyl carbons. More detailed assignments and conditions were as described in (Ralph *et al.*, 1998).

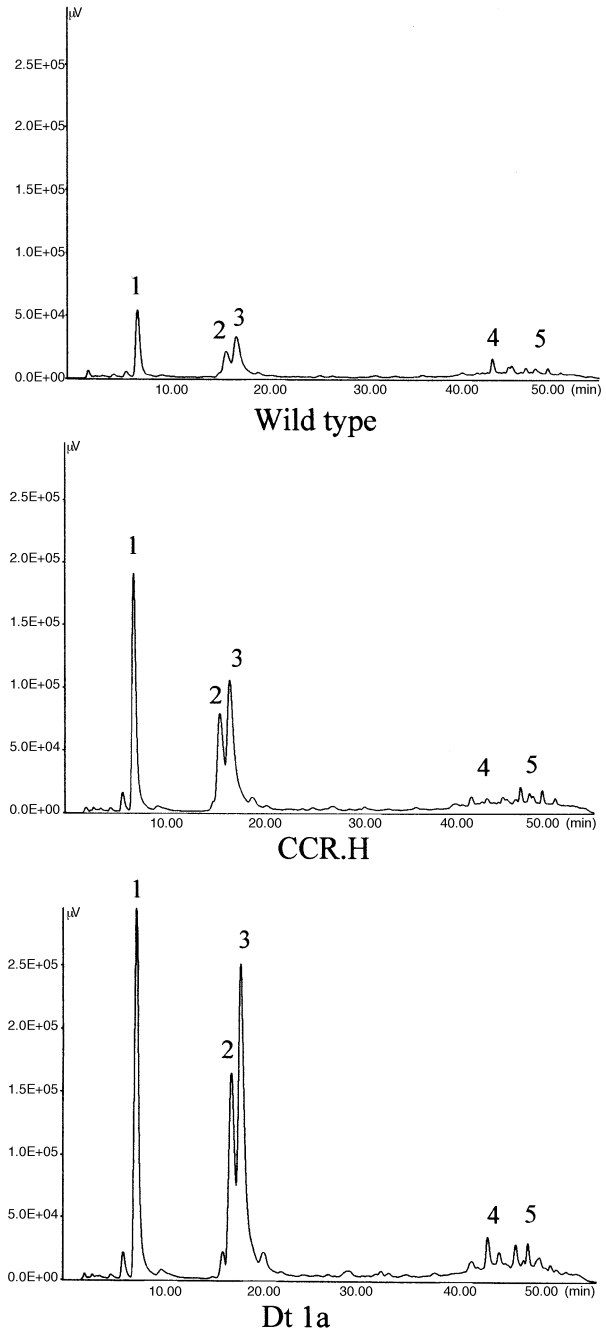


Figure 5. Separation and characterization of soluble phenolic compounds by HPLC. HPLC profiling of soluble phenolics performed on the oldest leaves of each tobacco (Wild type, CCR.H and Dt 1a: double transformant CAD/CCR). 1, 2, 3, 4 and 5 represent, respectively, neochlorogenic acid, 4-O-caffeoylquinic acid, chlorogenic acid, rutine and kaempferol-3-rutinoside. The HPLC profiles were obtained from the same amount of fresh material for the different lines.

(Figure 3c), signals from these units dominate the spectrum. With the concomitant incorporation of equivalently high levels of ferulate, the lignin is quite atypical and there is little surprise that thioacidolysis yields are also low; tying up lignin units with ferulates will limit the release of traditional thioacidolysis monomers. In the aldehyde region, the hydroxycinnamaldehydes are only minor peaks (in line with the reduced phloroglucinol staining observations) but the benzaldehydes levels are still substantial.

It is the lignin from the double transformant that is new and intriguing here. The NMR spectrum (Figure 3d), shows that the aldehyde signature is *not* typical of CAD-deficiency. In particular, there is no evidence for the 8-O-4-coupled hydroxycinnamaldehydes that are so prominent in the (solely) CAD.H deficient transgenics (compare Figure 3b). The CCR-deficiency signature, the presence of elevated tyramine levels, is clearly evident (although at a lower level than in the solely CCR.H deficient transgenic, Figure 3c), and again, as evident from the thioacidolysis data, the S + G yield is higher in the double transformant than in any of the other samples. The lignin in the double transformant therefore appears much more like the wild type than in either the CAD.H or CCR.H transgenics individually, as seen most readily by comparing the sidechain regions (50–90 p.p.m) of the spectra in Figure 3, although the tyramine levels are slightly elevated.

Other biochemical characteristics of transgenic plants

Other differential biochemical changes resulting from the potential reorientation to other sinks of the carbon flux normally directed to lignin synthesis, could explain the phenotypic differences between CCR.H and Dt (such as compensation mechanisms and changes in compounds with hormone-like activities). In this way we analysed the cell wall polysaccharides and the pools of soluble and wall-bound phenolic components of the transgenic lines with a reduced lignin content for comparison to the control.

Polysaccharides and non-lignin phenolic components of the cell wall. Table 2 shows the relative weight percentage of various neutral sugars recovered from acid hydrolysis of cell wall residues of the wild type, CCR.H and the double transformant (Dt 1a). The hydrolysis yield in xylose and glucose, which may, respectively, and more specifically originate from non-cellulosic polysaccharides and from cellulose, was substantially higher in the double transformant compared with the control. This increase was even greater for the CCR.H line strongly down-regulated for CCR.

In addition to neutral sugar analysis, we examined by GC–MS the low-molecular weight phenolics linked to the cell wall through alkali-labile bonds. As seen in Figure 4, different phenolic compounds are associated with the cell wall both for the CCR.H line and for the hybrid. Some of

the compounds such as sinapic and ferulic acids or acetosyringone are absent or in very low amounts in the wild-type and their relative increase is particularly pronounced for the transgenic lines (CCR.H and Dt 1a). However, no major qualitative differences were observed between the CCR.H line and the double transformant concerning the nature of the cell wall associated phenolics. Globally, these compounds were found to be less abundant in the double transformant (Dt 1a).

Soluble phenolic compounds. The phenolic profiles of mature leaves reported in Figure 5 show that the genetic transformations induced significant quantitative changes in the pools of different specific phenolics. Indeed, we found, respectively, three and six times more total soluble phenolic compounds in CCR.H and double transformant (Dt 1a) lines in comparison with the wild type. Among the peaks separated in the different lines it was possible to identify chlorogenic acid, 4-O-caffeoylquinic acid, neochlorogenic acid and also rutine and kaempferol-3-rutinoside. Chlorogenic acid was increased 2.7- and 6.2-fold, respectively, in CCR.H and double transformant (Dt 1a) in comparison with control plants and similar increases were found for neochlorogenic acid and 4-O-caffeoylquinic acid.

However, in our experimental conditions, no specific new structure can be identified in the double transformant (Dt 1a). The same trends for the same components were observed whatever the organs analysed, young leaves, apex or base of the stem.

Discussion

Simultaneous down-regulation of CCR and CAD induces specific changes in lignin content and composition

When CCR and CAD activities were both down-regulated, the transformed plants exhibited important changes in lignin content and composition. Lignin content was significantly decreased; almost 50% reduction was observed in certain crosses. These results contrast with those obtained for the CCR.h line hemizygous for the *ccr* gene, which exhibited a lower decrease in lignin content than the CCR.H homozygous line. We assumed that the difference observed between these lines (CCR.H and CCR.h) was due to a gene dosage effect as previously described in other cases (see for example Dai *et al*. (1999)). However, this mechanism cannot explain the results observed for the double transformant (Dt 1a) for which the activity reduction of the two enzymes seems not only to induce additive effects, but also to operate synergistically as far as lignin content is concerned.

CAD down-regulated plants compensate for the decrease in the synthesis of monolignols by direct incorporation of cinnamaldehydes in the polymer (Halpin *et al*., 1994; Kim *et al*., 2000; Yahiaoui *et al*., 1998). This compensation mechanism might apparently occur in the double transformants, as suggested by phloroglucinol staining, but is likely less marked than in CAD down-regulated plants since the supply of cinnamaldehydes is reduced. In fact the NMR spectra, Figure 3(d), show no accumulation of the 8-O-4-cross-products characteristic of CAD-deficiency. Apparently hydroxycinnamyl aldehydes are not exported to the walls in significant quantities in this line, although it is important to recognize that the lignins analysed represent only a fraction of the total lignin and that some partitioning may have occurred.

In addition to changes in the activities of target enzymes, the down-regulation of specific genes may have a specific impact on the expression of other genes of the lignification pathway. For example, CCR down-regulated *Arabidopsis* plants with phenotypic alterations similar to those observed for the CCR.H line displayed significant changes in the expression of different genes involved in the synthesis of lignin (*comt, cad*; Jouanin, pers. comm.). Moreover, we have recently shown, in collaboration with Legrand's group in Strasbourg, the impact of COMT down-regulation in tobacco plants on the expression of other genes of the pathway (Pinçon *et al*., 2001). It is then possible that in double transformants, the combined limited expression of both *ccr* and *cad* genes induces a specific modulation of other genes involved in the biosynthesis of lignins. The impact of metabolites from primary metabolism on gene expression is currently being increasingly demonstrated (e.g. hexoses: Jang and Sheen, 1994; methionine: Chiba *et al*., 1999) and these processes are also likely to occur for secondary metabolism. Cross-talk between lignification genes could occur *via* changes in specific phenolic compounds concentrations.

The simultaneous down-regulation of both genes did not significantly change the relative thioacidolysis yields (on a lignin basis) in contrast to the decrease observed for individual parental lines. Concerning the lignin monomeric composition, CCR silencing is associated with an increase in the S: G ratio and CAD silencing with a decrease in this ratio. The double transformants are closer to the CCR down-regulated plants with a relative enrichment in S units, which is generally considered to have positive effects on pulping characteristics (Chiang and Funaoka, 1990). However, in a general way, the resulting lignins, have a structure more similar to the wild-type control than either of the singly down-regulated transgenics. All together our results clearly show that the ectopic expression of a specific transgene may have different impacts depending on the genetic background. In the same way, Gentinetta *et al*. (1990) and Chabbert *et al*. (1994) have already reported variations in the effect of the bm3 mutation (affecting COMT) in different maize genetic backgrounds.

Plants with a severe reduction in lignin content may display a normal development and may maintain structural integrity of the vessels

Lignin deposition in cell walls has been a crucial step in the adaptation of plants to a land habitat. Consequently it is important to know to what extent it is possible to modify the lignin content and composition of plant cell walls without altering their normal development. Our initial studies on CCR down-regulation in tobacco plants (Piquemal *et al.*, 1998) suggested that decreased lignification could contribute to dramatic alterations in plant development. These observations underlined the potential limits of lignin genetic engineering experiments aiming at significantly decreasing the lignin content of plants. However, further results of Zhong *et al.* (1998) on the same plant material demonstrated that a very large decrease in lignin content was obtained without any significant changes in size or growth rate. These results were extended to poplar plants down-regulated for CCoAOMT, which could at least tolerate up to 40% reduction in lignin without major adverse effects on normal plant growth and development in the greenhouse conditions (Zhong *et al.*, 2000).

Similar conclusions were reached by Hu *et al.* (1999) on transgenic aspen down-regulated for a specific *4 cl* gene involved in lignification. In these plants a sharp decrease in lignin content was even associated with a significant increase in the growth of the plants. The results we observed for our tobacco hybrid plants down-regulated for both CCR and CAD are in agreement with these recent demonstrations and contrast with our previous studies on CCR down-regulated tobacco plants (Piquemal *et al.*, 1998). So, these data confirm that herbaceous or woody plants may, at least in controlled growth conditions, tolerate a strong reduction in their lignin content without severe modifications of their phenotype. In addition, these findings demonstrate that it is not the global reduction of lignin *per se* that is responsible for the reduced growth in our CCR.H lines but some specific associated effects which likely do not occur in the double transformants. Changes in one branch of the phenolic metabolism pathway can affect the metabolic flux in other pathways and it is known that the phenylpropanoid pathway generates a variety of plant compounds that are important for the adaptation and survival of plants. Indeed monolignol derivatives such as DCA (β– 5 dehydrodiconiferyl alcohol) or DCG (β– 5 dehydrodiconiferyl alcohol glucosides) have been shown to exert hormone-like activities and play a role in tobacco cell differentiation *in vivo* (Tamagnone *et al.*, 1998). Preliminary soluble phenolic profiling experiments have not allowed us to detect any qualitative differences between the different transgenic lines. However, these experiments have revealed the dramatic increase in soluble phenolics (mainly caffeic acid esters) occurring in the different transgenic lines when compared with the control. Meyermans *et al.* (2000) have also shown a dramatic increase in phenolic acid glucosides in the case of CCoAOMT down-regulated poplars, which display a slight reduction in lignin content. These various results confirm that a decrease in lignin content is logically associated with a reutilization of phenylpropanoid units in other branches of phenolic metabolism. These changes might, in certain cases, have an impact on developmental programs since phenolic compounds have, for a long time, been assumed to interfere with metabolism and function of plant growth substances. In addition, they could influence the interactions between the plant and its biotic environment and these potential repercussions should be envisaged in more detail in the future as they represent a still unknown facet of the effects of transgenesis.

Compensatory mechanisms are likely to occur to maintain vessel structural integrity in severely lignin depleted hybrid plants. Among these compensation mechanisms a relative increase in polysaccharide synthesis (already observed for transgenic aspen down-regulated for 4 CL) does not seem to play a crucial role in the double transformant since it was observed in a similar manner for the CCR.H line, which is strongly affected in its development and xylem structure. Furthermore, the double transformant (Dt 1a) has alkali-released phenolics similar to those of the CCR.H line. These data do not support different extents of cross-linking between the components of the wall according to the transgenic lines. One alternative hypothesis could be related to the structural differences observed between the lignins of the two transgenic lines with a decreased lignin content. The wild-type-like lignins of Dt could more efficiently maintain the supramolecular organization and the mechanical properties of the vessel walls than the dramatically altered lignins of the CCR.H line. Other mechanisms may possibly account for the differences observed in vessel integrity and particularly a selective decrease in lignification in specific cells of the xylem (Chabannes *et al.,* 2001).

Plants down-regulated for CCR and CAD activities may be interesting for industrial applications

The pulp industry is looking for plants containing less lignin or a lignin which can be more easily extracted. These two criteria are met individually by the CCR down-regulated plants and by the CAD down-regulated plants. However, as previously mentioned, the CCR down-regulated plants with a strong reduction in lignin content are not suited for practical exploitation since their development is dramatically affected. In contrast, the double transformants with a low activity of both CCR and CAD combine lower lignin content with a normal development.

Preliminary simulated pulping experiments performed on tobacco stems of these double transformants (Dt 1a) at the 'Centre Technique du Papier, Grenoble' (Petit-Conil *et al.,* pers. comm.) have revealed a significant decrease in kappa number, an increase in yield and in cellulose degree of polymerization. These data are in agreement with the dramatic modification of the cellulose/lignin ratio in the double transformant (Dt 1a). These characteristics are very attractive and plants should now be evaluated in different culture conditions, including field experiments, and the technology extended to woody species of economic interest. *4 cl* and *ccoaomt* genes have been suggested to be ideal targets for genetic manipulation of lignin in forage plants and trees since their down-regulation decreases the lignin content without altering the development (Zhong *et al.*, 2000). The combined down-regulation of CCR and CAD could also represent an alternative convenient strategy.

Experimental procedures

Plant material

Different tobacco lines (Wild type (Wt), single and double transformants) (*Nicotiana tabacum L.* cv. Samsun) were cultivated on vermiculite in a culture room (16 h/8 h 25°C/22°C with 80% humidity) or in a greenhouse for two and a half months and harvested just before flowering. All the determinations (unless otherwise stated) were carried out on the basal 5 cm of the stem of the different tobacco lines.

Transgenic tobacco lines

- CAD.H is a CAD down-regulated line homozygous for antisense *cad* cDNA (Halpin *et al.*, 1994)
- CCR.H is a CCR down-regulated line homozygous for antisense *ccr* cDNA (Piquemal *et al.*, 1998)
- CCR.h and CAD.h are the hemizygous lines resulting, respectively, from the backcross of CCR.H and CAD.H with the wild type line

  The four lines are kanamycin resistant.

Crossing procedure

Cross-pollination was performed when the flowers of the maternal plant were still closed (1 or 2 days before anthesis). After artificially opening the flower, the five stamens were gently taken off and the pollen of the father plant brought to the pistil.

Two different crosses were made:

- CAD.H♀ × CCR.H♂ (Dt 1a)
- CCR.H♀ × CAD.H♂ (Dt 1b)

PCR experiments and Northern blot analysis

The presence of both transgenes in the progeny was confirmed by PCR experiments using convenient primers after DNA extraction performed according to Edwards *et al.* (1991).

Northern blot analysis was performed as previously reported (Halpin *et al.*, 1998) using as homologous antisense DIG-labelled riboprobes, 0.6 kb of the *ccr* cDNA and 1.4 kb for the *cad* one. Extraction was performed from three pooled tobacco stems for each line.

Enzyme assays

The bottom of the stem (5 cm) of five plants of the same genotype was ground in liquid nitrogen and the proteins from these pooled samples, extracted at 4°C in 0.1 M Tris–HCl pH 7.5, 2% PEG 6000 (w/v), 5 mM DTT, 2% PVPP (w/v). The crude extract was clarified by centrifugation twice (10 000 g/10 min) at 4°C and used both for CCR and CAD activity measurements.

CCR activity was measured by a radiochemical method based on the conversion of ^{14}C-labelled feruloyl-CoA into ^{14}C coniferaldehyde. Radioactive [2–^{14}C]-ferulic acid was synthesized by standard Knoevennagel procedures (Semler *et al.*, 1987) using vanillin (Merck KGaA, Darmstadt, Germany) and 100 μCi [2–^{14}C]-malonic acid (NEN, Boston, MA, USA). Labelled ferulic acid was purified by preparative silica TLC using dichloromethane/EtOAc (95/5 v/v) as eluent. The yield was about 50% on the basis of the starting radioactivity. ^{14}C-Feruloyl coenzyme A was prepared according to the procedure described by Stöckigt and Zenk (1975). The intermediate ester (N-succinimidyl ferulate) was used without any purification for transesterification with coenzyme A (Pharmacia, Amersham Pharmacia Biotech, Piscataway, NJ, USA). ^{14}C-Feruloyl coenzyme A was purified by preparative cellulose TLC in butanol/acetic acid/water (5/2/3 v/v) and the yield was about 20%.

The radiochemical assay for the measurement of CCR activity was performed under the same conditions as the spectrophotometric assay (Piquemal *et al.*, 1998) but 12 000 dpm of labelled feruloyl-CoA were added to the reaction mixture (350 μl final volume). The enzymatic reaction was stopped after 10 min at 30°C by addition of 10 μl of 200 mM coniferaldehyde (dissolved in EMMEG) followed immediately by 1 ml of ethyl acetate. This medium was vortexed and centrifuged for 30 sec at 15000 rpm in a bench centrifuge. An aliquot (500 μl) of the organic phase containing only the reaction product was taken for radioactivity counting in a liquid scintillation apparatus (Packard, 1900 TR).

CAD enzyme activity was determined by measuring the increase in absorbance at 400 nm when coniferyl alcohol was oxidized to coniferaldehyde (Wyrambik and Grisebach, 1975). The assay was performed for 10 min at 30°C in a total volume of 0.5 ml containing 100 mM Tris–HCl (pH 8.8), 100 μM coniferyl alcohol, 200 μM NADP and 50 μl enzyme extract. For all the determinations, the values were expressed on a specific activity basis taking into account the amount of proteins in the sample. Protein concentration was determined by the method of Bradford (1976) using the dye-binding reagent supplied by Bio-Rad.

Microscopy

Fluorescence microscopy and cell imaging. Free-hand sections were mounted on a glass slide and observed using an inverted microscope (Leitz DMIRBE, Leica, Heidelberg, Germany) equipped with epifluorescence illumination (ultra-violet excitation range, with either a BP340-380 excitation filter and an LP 430 suppression filter, or BP450-490 excitation filter and an LP520 suppression filter). Images were acquired using a CCD camera (Colour Coolview, Photonic Science, Millham, UK).

Histochemical analysis. Hand-made stem sections were cleared by sodium hypochlorite treatment (1.25% of active chloride) and stained with phloroglucinol-HCl (Wiesner reagent;

Adler *et al.*, 1948). Stained sections were observed using an inverted microscope (Leitz DMIRBE, Leica).

Sample preparation and lignin analysis. Whole stems from control and transgenic lines were frozen in liquid nitrogen. After freeze-drying (48 h), the woody xylem ring from five plants of the same line was manually removed from the pith and cortex, pooled, ball milled to a fine powder and sequentially extracted with water, ethanol and toluene:ethanol (1/1: v/v) using a modified Soxhlet apparatus (Perstorff Instruments, Tecator-Perstorp Analytical, Hoganas, Sweden). The resulting cell wall residue (CWR) was rinsed with acetone, dried and analysed using a variety of different methods. All the results shown, unless otherwise stated, are the mean value obtained from four independent experiments performed on the same starting material (five independent pooled plants). Klason lignin contents were determined using a micro-Klason technique (Whiting *et al.*, 1981). The monomeric yield and composition of the non-condensed fraction of lignins (i.e. monomers linked exclusively through (β– O-4 ether bonds) were determined after thioacidolysis, according to the method of Lapierre *et al.* (1986).

Isolation of lignins and NMR. Lignin was isolated from an entire stem of each ^{13}C-enriched tobacco (*Nicotiana tabacum* L) line including wild-type, CAD (CAD.H), CCR (CCR.H), and CCR/CAD (Dt 1a). ^{13}C-enrichement for NMR purposes was by growing plants in ^{13}C-enriched CO_2 atmopheres as described by Ralph *et al.* (1998). Lignin isolations were essentially as described by Marita *et al.* (1999). Soluble phenolics, carbohydrates and other components were removed by successive extractions with water, methanol, acetone, and chloroform. The isolated cell walls were ball-milled, suspended in acetate buffer and treated with Cellulysin cellulase before extraction with 96 : 4 dioxane:water reflective of standard 'milled wood lignin' conditions (Björkman, 1956). The soluble fraction was then filtered to remove water-soluble components mainly low molecular weight sugars. The amounts isolated (% of the cell wall material) were WT: 6%; CAD.H: 14%; CCR.H: 1.5%; Dt 1a: 4%. Ball-milling of the CCR sample was problematic. Due to the low amount of material, iron filings from the ball mill contaminated the sample and prevented accurate measurement of weights and recoveries. Isolated lignins were acetylated overnight with acetic anhydride/pyridine, and the solvents were removed by co-evaporation with 95% ethanol. Traces of ethanol were removed by co-evaporation with acetone. The acetylated lignins were then extracted into $CHCl_3$ and washed with 6 mM EDTA (pH 8.0) to remove any trace of metal contaminants. The acetylated lignins were dissolved in acetone-d_6 (400 μl) for NMR.

Alkaline hydrolysis and characterization of cell-wall bound phenolics. The extraction procedure from 10 mg of CWR and the GC–MS analysis of the low molecular weight phenolics as their trimethylsilyl derivatives (TMS) were performed according to Jacquet *et al.* (1995). The GC–MS identification was performed in comparison with authentic standard compounds. The GC–MS quantitative determination was run after calibration with standard compounds and from ion chromatograms reconstructed on the molecular ion of the various TMS derivatives. The variability between analyses were in the 10–15% range.

Extraction and analysis of total soluble phenolic compounds. Plant material of four different origins (0.5 g) (young leaves, mature leaves, apex of the stem and base of the stem) was ground in liquid nitrogen and the soluble compounds extracted three times at 4°C in 50 ml of 80% ethanol. The crude extract (150 ml) was filtered and evaporated at 35°C under reduced pressure. The aqueous phase was depigmented by gentle shaking twice with petroleum-ether (40–60°C boiling fraction). The aqueous extract was freeze-dried and stored at –20°C until analysis.

Analysis of soluble phenolic compounds by HPLC

The previous freeze-dried material was dissolved in 0.5 ml water and filtered prior to injection into the HPLC or HPLC-MS system.

The extract was first analysed by HPLC in a 60-min run on a C18-silica column (Ultrasep, 6 μm, 25 cm long, 4 mm diameter, Bischoff) in a Spectra Physics HPLC apparatus with an SP 8800 ternary pump and Spectra UV 100 detector. The flow rate was 1 ml min^{-1} and the column was maintained at 35°C. Phenolics were detected at 340 nm. Solvents were as follows: solvent A, methanol/water/acetic acid 10/88/2; solvent B, methanol/water/acetic acid 88/10/2.

The gradient was: 100% solvent A at time 0, 70% solvent A and 30% solvent B at 35 min, 34% solvent A and 66% solvent B at 50 min, 100% solvent B at 55 min.

Identification of components by HPLC-MS. Compounds were identified by coinjection of standards (chlorogenic acid, rutine and kaempferol-3-rutinoside) and comparison with reported elution orders (Snook and Chortyk, 1982). To confirm the HPLC data, the extracts were analysed by HPLC-electospray ionization-MS. The system used for HPLC-MS analysis was a Finnigan LCQ mass spectrometer equipped with a Spectra System P1500 HPLC pump and a Spectra System UV 1000 detector (Spectra Physics, Les Ulis, France). The capillary heating and voltage were maintained at 200°C and 4.50 kV.

For the quantification of the main compounds (neochlorogenic acid, 4-O-caffeoylquinic acid and chlorogenic acid), the response factors for neochlorogenic acid and 4-O-caffeoylquinic acid (both isomers of chlorogenic acid) were assumed to equal that of chlorogenic acid. Peak areas were determined and concentrations of the components calculated by reference to the calibration of the peak areas of chlorogenic acid from parallel runs.

Qualitative analysis of polysaccharides (neutral sugars)

Sulphuric acid hydrolysis of CWR, followed by GC of the released sugars (analysed as their alditol acetates) was performed according to standard protocols (Blakeney *et al.*, 1983; Englyst and Cummings, 1984). The results are expressed as weight percentages of the various neutral sugars recovered from the samples and after calibration with authentic compounds.

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Abbreviations

COMT: Caffeic acid/5-OH ferulic acid O-methyltransferase
CCoAOMT: Caffeoyl coenzyme A 3-O methyltransferase
4 CL: 4-coumarate CoA ligase
F5H: Ferulate 5-Hydroxylase
CCR.H: Transgenic tobacco line homozygous for the *ccr* antisense gene
CCR.h: Transgenic tobacco line hemizygous for the *ccr* antisense gene
CAD.H: Transgenic tobacco line homozygous for the *cad* antisense gene
CAD.h: Transgenic tobacco line hemizygous for the *cad* antisense gene
Dt: Double transformant down-regulated for both CCR and CAD

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